Cerebellar Development Requires Cyclin A2

Research Thesis

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Abstract

Cyclins regulate transitions through the cell cycle by activating cyclin-dependent kinases to phosphorylate other enzymes into an active state. Cyclin A2 regulates S-phase transitions, and is observed in two isoforms: a full-length ("A60") form and a truncated ("A38") form. The goal of this study was to determine the function of *cyclin A2* in proliferating neural progenitor cells. To achieve this goal, we performed a developmental analysis of mouse brains for the presence of Cyclin A2. Cyclin A2 was identified in proliferating cells of the developing cerebellum as well as in neurons of the adult cortex. Western blotting determined that A60 form of Cyclin A2 in the developing cerebellum, while the A38 form was found in differentiated areas of the brain including the adult cerebellum and cerebral cortex. We concluded that A60 predominates during cerebellar growth whereas the A38 form predominates during cerebellar maturation, suggesting that proliferating neural progenitor cells are characterized by the A60 form whereas differentiated cells are characterized by the A38 form. Cre/loxp-based deletion of *cyclin A2* in neural progenitors was lethal and resulted in cerebellar hypoplasia, decreased proliferation of cerebellar granule neuron progenitors, and Purkinje neuron dyslamination. To assess a potential mechanism by which *cyclin A2* promotes normal cerebellar structure, we examined the size of the cerebellar external granule layer (EGL). At embryological day 14.5, the EGL showed a significant decrease in volume in *cyclin A2*-null cerebella compared to controls. Furthermore, we observed a large increase in apoptotic cells in the EGL. These differences were not observed in older embryos, indicating that early loss of cells of the EGL leads to the observed cerebellar defects. These data show the vital role *cyclin A2* has in cerebellar morphogenesis.

Introduction

The central nervous system (CNS) develops through the regulated proliferation of neural progenitor cells. Cell proliferation is driven by cyclins and cyclin-dependent kinases (CDKs) (Bloom and Cross, 2007). Together, cyclins and CDKs form complexes to regulate various cell cycle phases in conjunction with other proteins. In the original model, specific cyclins/CDK complexes regulate transitions through the G1, S, G2, and M phases of the cell cycle. Recent experiments demonstrating redundancy in cyclin-CDK binding and cell cycle progression have challenged the original model (Aleem et al., 2005). Cyclins have also shown to regulate other processes including CNS synapse development, including synaptic plasticity, memory formation (Odajima et al., 2011), and DNA repair (Jirawatnotai et al., 2011).

The mammalian genome encodes two A-type cyclins, *cyclin A1* and *cyclin A2*. Testis-specific *cyclin A1* is restricted to germ cells (Sweeney et al., 1996) and necessary for male meiosis (Liu et al., 1998) whereas *cyclin A2* is ubiquitously expressed in all proliferating cells (Yang et al., 1997a) and is required for the onset of DNA replication (Girard et al., 1991). Analyses of conventional *cyclin A2* null mice showed that these animals failed to survive past embryonic day 5.5, emphasizing the critical role *cyclin A2* has in cell proliferation (Murphy, 1999). A conditional ablation of A-type cyclins revealed that *Cyclin A2* function is essential for cell cycle progression of hematopoietic and embryonic stem cells, yet redundant with *cyclin E1* in mouse embryonic fibroblasts (Kalaszcynska et al. 2009).

The cerebellum is derived from two germinal zones, the ventricular/subventricular zone surrounding the fourth ventricle, and the external granule layer (EGL) derived from the rhombic lip. CGNP emerge from the rhombic lip and then migrate through the molecular layer to establish the internal granule layer (Altman and Bayer, 2008) while Purkinje (PC) neurons originate in the hindbrain ventricular/subventricular zone (Hatten and Heintz et al., 1995; Ten Donkelaar et al., 2003) and develop to become a source of Sonic hedgehog (SHH) proteins (Wallace 1995). SHH signaling regulates cerebellar morphogenesis (Dahmane and Ruiz I Altaba, 1999) and CGNP proliferation via the expression of Nmyc. *Nmyc* is a direct Shh transcriptional target (Kenney et al., 2003) and is the main downstream effector of the Shh pathway during the expansion of CGNPs (Hatton et al., 2006) While *cyclin A2* expression can be induced by SHH signaling in CGNPs (Zhao et al., 2002), *cyclin A2* function in forebrain and cerebellar development is not known. This study investigates the role of *cyclin A2* during CNS development through a gene targeting approach in neural precursor cells .

Materials and Methods

Transgenic Mice and Animal Husbandry

The generation of conditional *cyclin A2* $^{fl/fl}$ knockout is described by Kalaszczynka et al., and references therein (Kalaszczynka et al., 2009). *Cyclin A2* $^{fl/fl}$ mice were bred to *nestin-cre* mice to target neural pregenitor cells (Tronche et al., 1999). *Nestin-cre* mice show recombination in the CNS, including all cells in the cerebellum by embryonic day 13.5 (Huang et al., 2010) and adult Purkinje cells (Jennemann et al., 2005). Verification of *cyclin A2* knockout was performed by western blot analysis (Otero et al., 2014). Developmental analyses were performed in the CD-1 mouse strain (Charles River). All

animal experimentation was performed in complete compliance with the institutional review board requirements of Dana Farber Cancer Institute, University of California, San Francisco, and The Ohio State University.

Genotyping and Polymerase Chain Reaction

DNA was extracted from 5 mm-long tissue samples from the postnatal mice, or an equal amount of placenta from prenatal mice. Samples were put into sterile Eppendorf tubes individually, digested in 500 uL of digestion buffer (50mM tris(hydroxymethyl)aminomethane(TRIS), 100mM ethylenediaminetetraacetic acid (EDTA), 100mM sodium chloride, 1% sodium dodecyl sulfate (SDS), 0.5 mg/ml Proteinase K) for 24-36 hours in 55°C. After incubation, 500uL phenol:chloroform:isoamylalcohol (25:24:1) was added to each tube, mixed by repeated inversions and centrifuged at 18000 x g for 1 minute. The top aqueous phase was transferred into a new tube and precipitated with 20uL 5M NaCl and 1mL 100% ethanol. The suspension was mixed by vortexing and centrifuged at 18000 x g for 1 minute. The supernatant was removed by pipetting and pellet was resuspended with 500 uL of TE. A second extraction was performed with 500 uL of chloroform followed by ethanol precipitation. The DNA pellet was washed with 70% ethanol and centrifuged again (18000 X g for 1 min.). Supernatant was removed, DNA pellet was air dried for 5-10 minutes and resuspended in 100 uL of 1x TE buffer. DNA samples were used for detection of *cyclin A2* loxP alleles using primers 5'GTCTTGTGGACCTTCACCAGACCT3' and 5'GTACAGCATGGACTCCGAGCGAC3' and *cre* using primers 5'AATCGCGAACATCTTCAGGT3' AND 5'AAAATTTGCCTGCATTACCG3'. PCR mixtures containing 1uL of DNA, 0.375 uL of

each primer, 12.5 uL PCR direct Red Dye premix (Clontech 639286) and 10.375 uL of sterile deionized water were subjected to PCR amplification. Upon completion of PCR, samples were analyzed on a 1.5% agarose gel electrophoresis at 100 volts for 1 hour. Expected bandwidths are present at 700 bp for *cyclin A2* $^{fl/fl}$, 400 bp for cyclin A2 $^{+/+}$, and 200 bp for *nestin-cre.*

Immunofluorescent staining

To evaluate Cyclin A2 protein localization, wild-type mice were sacrificed at embryonic day 11, post-natal day 7 and adult ages, and heads or brains were dissected and drop-fixed in 4% paraformaldehyde overnight then incubated in 30% sucrose-1X PBS at 4°C for 24-36 hours. Tissues were embedded in OCT and cryosectioned at 12-14 μm.

The following primary antibodies were used for immunohistochemistry: GFAP (Abcam, ab7777-500), cleaved Caspase 3 (Cell Signaling, 9664S), Ki67 (Vector Labs, VP-RM04), phosphistone H3 (Cell Signaling, 2650S), TUJ1 (Covance, MMS-436 P-250), and cyclin A (Santa Cruz, SC-596). Appropriate secondary antibodies were purchased from Invitrogen/Molecular Probes. Confocal microscopy instrumentation included Axio ImagerZ2 with LSM710 module.

Unbiased Stereology

To determine estimates of volume and total number of cleaved-caspase 3 positive cells in the embryonic cerebellum and forebrain using StereoInvestigator (MBF Biosciences), experimental and control mice were sacrificed at E14.5 and E17.5 and embryos were dissected and drop-fixed in 4% paraformaldyde overnight then incubated in 30% sucrose-1X PBS at 4°C for 24-36 hours. Embryos were cryosectioned at 50 μm

and every 5th section was immunostained with cleaved-caspase 3 (Dako Envision kits, K400711-2 and counterstained with hematoxylin (Fisher Scientific CS400-4D) and coverslips were mounted with permount (Fisher, SP15-500). Cavalieri estimations generated volume estimates with the following parameters: grid size = 30 μm, shape factor = 4. Cleaved caspase-3 total cell number estimates were obtained using optical fractionator probes with the following parameters: counting was performed under oil immersion with 100x objective, dissector height = 20 μm, dissector volume = 50,000 μm2, counting frame height and width = 50 μm, sampling grid was 153.9 μm x 162.5 μm. The term ventricular/sub-ventricular zone (VZ/SVZ) refers to the 4th ventricular germinal neuroepithelium of the cerebellum or lateral ventricular germinal neuroepithelium of the forebrain, which for quantification purposes includes the dense population of neural progenitor cells lining the 4th ventricle or lateral ventricles.

Results

Cyclin A2 protein localizes to neural progenitor cells during CNS development

To investigate the role *cyclin A2* in CNS development, we performed a developmental analysis on CD-1 wild-type mice at a spectrum of developmental ages. Cyclin A2 protein can be observed in two isoforms, a full-length “A60” form and an N-terminal truncated “A38” form (Welm et al., 2002). During the proliferative ages in the cerebellum, the A60 form is mainly observed, after which the A38 takes over. In contrast, the A38 predominates during the post-natal ages throughout development (Otero et al., 2014). To test which cells express *cyclin A2* during CNS development, we performed confocal immunofluorescent microscopy of cyclin A2 expression in the CNS of CD1 mice at E11, P7, and adult ages (**Fig. 1**). At E11 in the SZ/SVZ of the 4th, we

noted cyclin A2 expression in Ki67 positive cells and pH3 positive cells, with some processes oriented apically towards the pial surface and negative cyclin A2 expression in TUJ1 positive cells and GFAP positive cells. In the P7 cerebellum, cyclin A2 was seen in mitotic cells and cerebellar neuron progenitor cells that were negative for TUJ1 and GFAP. The adult cerebellum showed cyclin A2 colocalization in the neuronal somata and nuclei of PC cells and neuropil in the internal granule layer (IGL) while cyclin A2 colocalization was seen only in nuclei and soma of cortical neuron in the adult cortex. We conclude that during neurogenesis, a period characterized primarily by the A60 form, proliferating cells express cyclin A2 whereas adult neurons, characterized by A38 predominace, express nuclear and cytoplasmic cyclin A2.

Rhombic lip-derived and VZ/SVZ-derived neural progenitor cells show differential requirements to cyclin A2

To test if cerebellar development requires *cyclin A2* at earlier embryonic ages, we evaluated EGL volume and programmed cell death in E14.5 and E17.5 embryos and 4tth ventricle VZ/SVZ volume and programmed cell death at E14.5 (**Fig. 2**). Volumes were calculated using Cavalieri estimations, and total cleaved caspase-3 positive cells were quantified by optical fractionator. The E14.5 VZ/SVZ of the 4th ventricle showed no significant change in volume or number of cleaved caspase-3 cells in *nestin-cre, A2* $^{fl/fl}$ compared to *cyclin A2* preserved littermates. In E14.5 embryos, EGL total volume was reduced approximately 40% in *nestin-cre A2* $^{fl/fl}$ mice and showed a significant increase in the number of cleaved caspase-3 positive cells. The E17.5 embryos continue the trend of decreased EGL volume and increased number of cleaved caspase-3 positive cells but did not meet the threshold for statistical significance.

The above findings suggested to us that other proliferative zones would show similar differentiation requirements to *cyclin A2* during development. To test this hypothesis, we evaluated the cortical plate and VZ/SVZ of the lateral ventricles volume and programmed cell death in E14.5 and E17.5 embryos (**Fig. 3**). At E14.5, the cortical plate and VZ/SVZ of the lateral ventricle showed a significant decrease in volume and number of cleaved caspase-3 positive cells in *nestin-cre, cyclin A2* $^{fl/fl}$ compared to *cyclin A2* preserved animals. In contrast, E17.5 *nestin-cre, cyclin A2* $^{fl/fl}$ embryos resulted in a significant increase in cleaved caspase-3 positive cells but the *cyclin A2* preserved mice showed no change in volume. We conclude that *cyclin A2* loss in embryonic cerebral VZ/SVZ, cortical plate, and CGNPs induces increased programmed cell death in the forebrain and cerebellum, whereas *cyclin A2* loss in embryonic cerebellar VZ/SVZ neural progenitor cells does not induce programmed cell death.

Discussion

Cyclin A2 regulatory functions in EGL proliferation, cerebellar foliation and cortical lamination

The cyclin A2 cerebellar phenotype is different from other reported cyclin-type ablations. The loss of *cyclin D1* leads to diminished post-natal cerebellar growth, diminished PC neuron dendritic arborization, and milder CGNP proliferation (Pogoriler et al., 2006). On the other hand, although *cyclin D2* null mice have decreased EGL proliferation, they do not show as severe abnormalities in PC dendritic arborization or cortical lamination, and Bergmann glial fibers extend to the sub-pial surface (Huard et al., 1999). Although simultaneous loss of two D-type cyclins results in a small

cerebellum, these mice show a better formed folia and minimal PC displacement into the molecular layer (Ciemerych et al., 2002).

The cerebella of *cyclin A2* null mice show more drastic cytoarchitectural abnormalities including abnormalities in cell layer, and patches of densely packed and dyslaminated PC cells (Otero et al., 2014) suggesting its critical function downstream of SHH signaling. SHH treatment upregulates *cyclin A2* in CGNPs (Zhao et al., 2002) and *cyclin A2* is highly expressed in medulloblastoma (Thompson et al., 2006). Several studies show that cerebellar SHH loss decreases cerebellar folia and induces PC dsylamination (Dahmane and Altaba, 1999; Wechsler-Reya and Scott, 1999) to a similar degree as in *cyclin A2* null mice. Seminal experiments also show that prevention of EGL proliferation by brain irradiation leads to a smaller cerebellar size and PC dsylamination (Altman and Anderson, 1971). However, it is unclear whether these abnormalities in cerebellar lamination are due to absent SHH morphogen signaling or a consequence of decreased CGNP proliferation independent of SHH's morphogen activities. To shed some light on these questions, we targeted the *cyclin A2* allele using a cre/loxp targeting approach. Our data show that reduced EGL proliferation and increased apoptosis intrinsically alters cerebellar development, and suggests that the post-natal dysmorphic cerebellar phenotype may be due to a combination of early embryonic cerebellar apoptosis in EGL and reduced EGL proliferation.

Conclusion

We tested the hypothesis that forebrain and cerebellar development requires *cyclin A2* utilizing a conditional knockout gene target approach. During embryonic development, conditional *cyclin A2* null mice showed increased programmed cell death

and a significant decrease in cortical plate and VZ/SVZ of the lateral ventricles, and EGL volume. These data show the novel role *cyclin A2* has in early embryonic forebrain and cerebellar morphogenesis.

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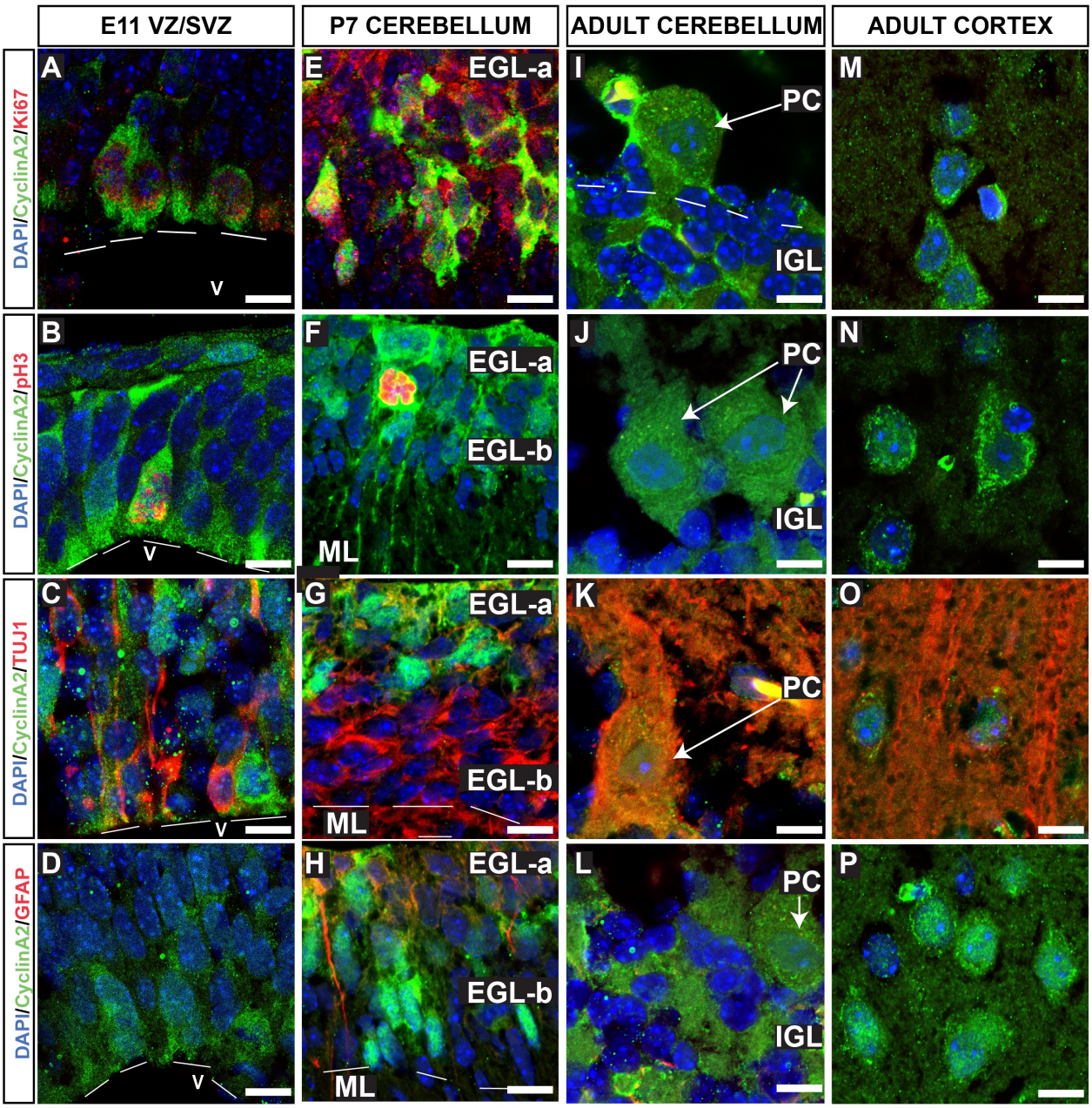

Figure 1: Confocal microscopy of wild-type mouse brains from cryosections stained for Cyclin A2 in cells undergoing cell cycle (Ki67), mitotic cells (pH3), neurons (TUJ1), and astrocytes (GFAP) and counterstained with DAPI to visualize nuclei. *Scale* bars = 10 μm, EGL = External Granule Layer, PC = Purkinje Neurons, IGL = Internal Granule Layer, ML = Molecular Layer, V = Ventricle.

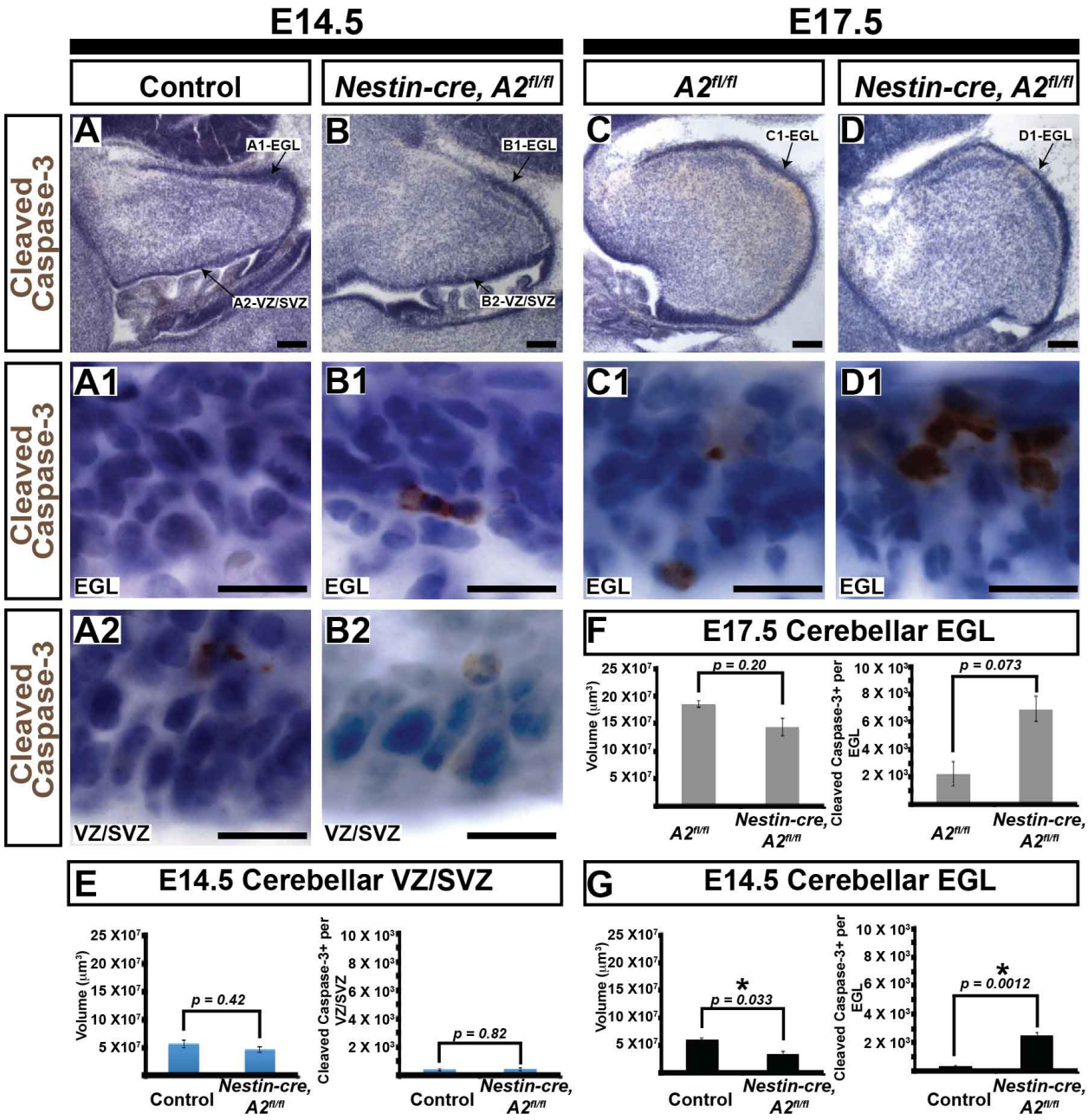

Figure 2: At embryonic day 14.5, the EGL showed a statistically significant increase in cleaved-caspase 3 positive cells and a reduction in volume but no significant change in VZ/SVZ of the 4th ventricle and EGL at embryonic day 17.5. Scale bars in A, B, C, D = 100 µm, scale bars in A1, A2, B1,B2, C1, D1 = 12.5 µm, EGL = External Granule Layer, VZ/SVZ ventricular zone/ subventricular zone in the 4th ventricle, *denotes statistical significance, P values determined by 2-tailed homoscendastic T-test.

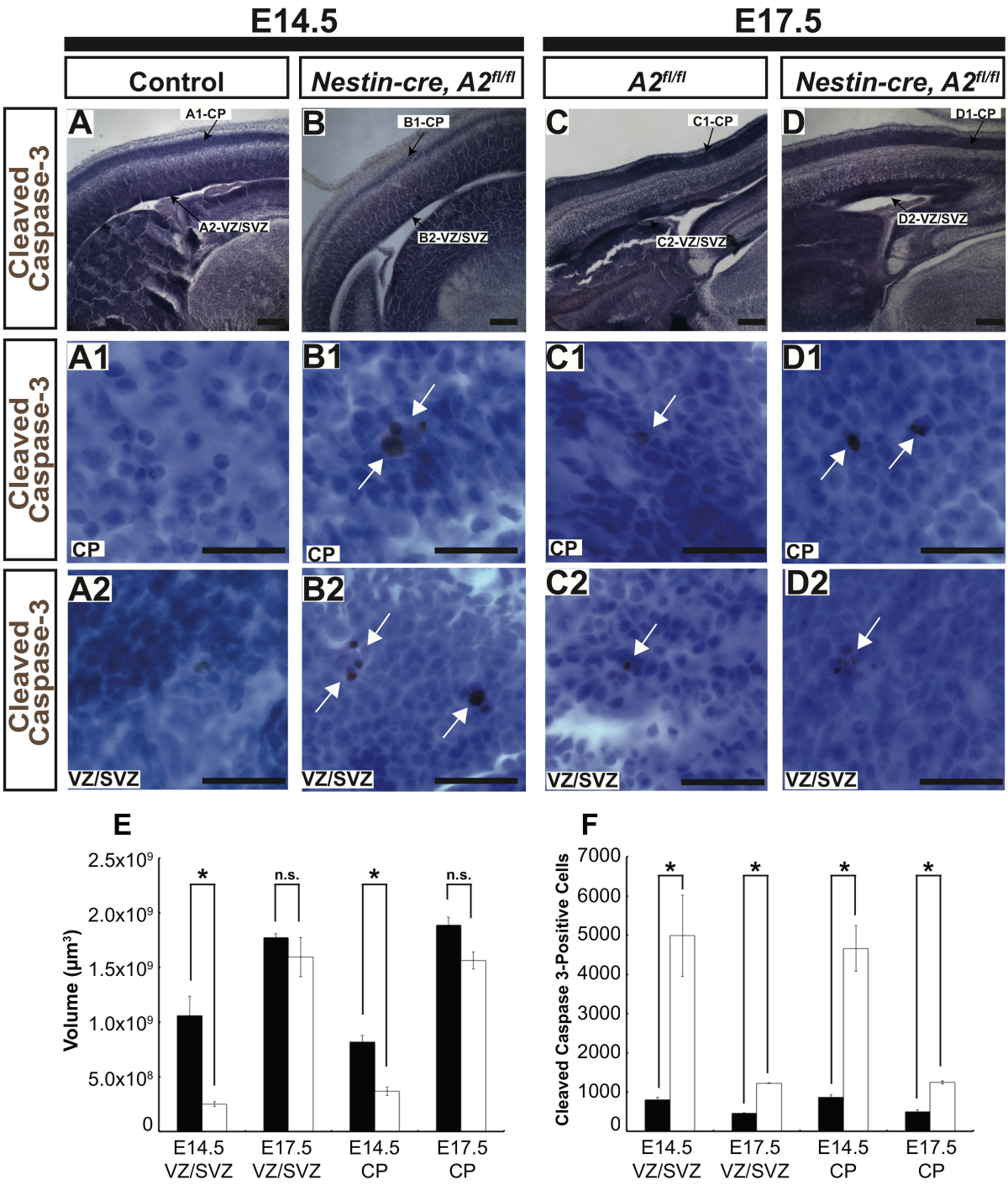

Figure 3: At embryonic days 14.5 and E17.5 in nestin-*cre, A2* $^{fl/fl}$ animals (shown in white), the cortical plate and VZ/SVZ of the lateral ventricle showed a statistically significant increase in cleaved-caspase 3 positive cells and a reduction in volume. Scale bars in A, B, C, D = 100 µm,

scale bars in A1, A2, B1,B2, C1, D1 = 12.5 μm, CP = Cortical Plate, VZ/SVZ ventricular zone/ subventricular zone of the lateral ventricles, *denotes statistical significance, P values determined by 2-tailed homoscendastic T-test.

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